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34. (Amended) A method according to claim 33, wherein all possible single-base mutations for a single codon in a single target nucleotide sequence, or all possible single-base mutations for two or more [nearby or] adjacent codons, or at nucleotide positions which require overlapping oligonucleotide probe sets, in multiple target nucleotide sequences are distinguished, the oligonucleotide probe groups containing oligonucleotide probes with target-specific portions which overlap.

81. (Twice Amended) A method ~~according~~ to claim 80, wherein each capture oligonucleotide differs ~~from its~~ adjacent capture oligonucleotide on the array by at least [25%] one out ~~of~~ every four of the total number of nucleotides when the oligonucleotides are aligned end to end with one another without internal insertion or ~~deletion~~.

## REMARKS

The July 16, 1998, personal interview between Examiners Adams, Ricigliano, and Fuldner, applicant Francis Barany, and applicants' undersigned attorney is gratefully acknowledged. The substance of the interview largely follows the remarks of the June 26, 1998, amendment. However, it was also requested that applicants demonstrate why it would not have been obvious to combine prior art ligase detection reaction technology with the prior art DNA array technology to arrive at the present invention. The following remarks as well as the accompanying Declaration of Francis Barany Under 37 CFR § 1.132 ("Barany Declaration") are submitted to respond to this issue.

The ligase chain reaction and ligase detection reaction procedures are well known in the art and have been shown to be useful for detection of genetic diseases where a genetic defect is either not present (i.e. normal individuals), present in 50% of the DNA (i.e. heterozygous individuals), or always present (i.e. homozygous mutants) (Barany Declaration ¶¶ 5-6). However, the situation is more complex when detecting cancer and cancer associated mutations where, in primary tumors, signal may be as low as 15% of wild type DNA, while, in early detection of cancer metastasis, signal may range from 1% to 0.1% of wild type DNA (Barany Declaration ¶ 6). In addition, many genes may be responsible for cancer development (Barany Declaration ¶ 8). For example, in colorectal cancer, the three most commonly mutated genes are the APC, p53, and K-*ras* genes, where there are

mutations, respectively, present in 70%, 50%, and 40% of colorectal cancers. (Id.) Moreover, different codons within each gene may be mutated (Id.) In view of these problems, there is an urgent need to develop nucleic acid based procedures for cancer detection (Id.).

A number of DNA array-based technologies are known in the art; however, they are not satisfactory for cancer detection. As shown in paragraphs 9-10 of the Barany Declaration, arrays have been proposed to be useful for quantifying expression level, and determining sequences of short nucleic acids. However, the incorrect hybridization of target nucleotide sequences to capture probes on arrays can result in sequencing errors (Barany Declaration ¶ 12). A great amount of effort has been expended to avoid such errors; however, approaches to avoiding hybridization errors on arrays are limited, have high false positive rates, are susceptible to high background noise, are not useful for detecting sequences likely to be present in a sample in small amounts, such as cancer mutations (Barany Declaration ¶¶ 10, 13-15, and 18).

The present invention's use of PCR/LDR in conjunction with array capture of ligation products constitutes a significant advance over the art, particularly with regard to cancer detection. For example, this procedure has been found to be useful in identifying all 19 possible single-base mutations in K-*ras* codons 12, 13, and 61, with a sensitivity of 1 in 500 wild type sequences (Barany Declaration ¶¶ 19-23 and 26). Thus, the multiplex PCR/LDR procedure of the present invention succeeds where dideoxy sequencing or array hybridization would fail (Barany Declaration ¶ 23). Although there are PCR-based techniques to detect single-base mutations present in a minority population of human tumor cells, these procedures are unsatisfactory due to mis-extensions and polymerase errors which cause false positive signals; they are also unable to detect the full spectrum of mutations in a given gene locus (Barany Declaration ¶ 24).

The present invention is able to overcome the deficiencies of the prior art by separating the amplification (i.e. PCR) and mutation discrimination (i.e. LDR) steps (Barany Declaration ¶ 25). The advantages of PCR/LDR include the ability to carry out large scale multiplexing, quantitative detection in a high background at normal sequences, detection of closely clustered mutations, and automated detection (Barany Declaration ¶ 26). High throughput is achieved by providing the LDR probes with addressable portions to guide ligation products to specific sites on an array (Barany Declaration ¶ 27). By uncoupling

polymorphism identification from hybridization, each step can be optimized independently (Id.). This is particularly useful in quantitatively assessing allele imbalance while achieving a high signal to noise ratio (Barany Declaration ¶¶ 27-28). As shown in paragraphs 29-32 of the Barany Declaration, an example of this is the use of PCR/LDR to detect mutations in codons 12 and 13 of the K-*ras* gene on an array. In using the combination of PCR/LDR and a DNA array, in accordance with the present invention, to detect K-*ras* gene mutations: (1) hybridization signal was correct for all nine test arrays; (2) a signal to noise ratio of greater than 3 to 1 was achieved (despite the expectation that unligated LDR probes would out-compete ligation products for array capture sites); and (3) quantifiable results were achieved even when target mutant DNA was present in a 20-200 fold excess of wild-type sequence (See Barany Declaration ¶¶ 29-32). Thus, the present invention is useful in detecting low abundance cancer mutations (Barany Declaration ¶ 32). This is not possible with prior techniques involving arrays where the error rate in assembly of capture oligonucleotide probes is so high that there will not be sufficient addresses to permit capture of target present in small amounts (Barany Declaration ¶ 33). Such prior art experience teaches away from the present invention and, indeed, demonstrates that the results achieved with applicants' invention are unexpected (See id.).

U.S. Patent No. 5,415,839 to Zaun et al. is fully discussed in the June 26, 1998, amendment. Applicants simply wish to emphasize that the entire focus of this reference is the amplification of target nucleotides--not the detection of single base differences, as claimed. Indeed, as pointed out in the prior amendment, with regard to the sickle cell anemia situation, Zaun's suggestion that gap-LCR can be employed forecloses any assertion that Zaun is to be used to detect single base differences.

U.S. Patent No. 5,695,934 to Brenner ("Brenner") discloses a procedure for massive parallel sequencing of different fragments of DNA. This technique involves (1) cloning fragments of DNA and sorting them onto beads or a two dimensional array; (2) sequencing the fragments by repeatedly labeling the fragments, identifying what base the label corresponds to, and removing one nucleotide from the fragment; and (3) assembling the fragments into a completed contig (Barany Declaration ¶ 16). Brenner's step (1) involves a combinatorial approach to synthesizing tags on beads where each bead has identical tags which differ from the tags on other beads (Id.). Both addresses and their complements are synthesized, and a portion thereof are cleaved from the beads and hybridized with each other

to make the tags double stranded (Id.). The tag complements have minimally cross-hybridizing properties (Id.). The oligonucleotide tags are ligated to fragments of polynucleotides to be sequenced, placed into a vector, and cloned into *E. coli* (Id.). The *E. coli* is then permitted to grow so that numerous copies of this identical bacteria with copies of the oligonucleotide tag and the polynucleotide fragment are produced (Id.). The joined oligonucleotide tag and polynucleotide fragment from each colony is excised, and a biotin or *Fok*I restriction site is then placed at the end of this fragment which does not have the tagged oligonucleotide to facilitate the sequencing procedure of step (2) (Id.). The tagged oligonucleotide is then made single stranded by digestion with a polymerase enzyme having 3' to 5' exonuclease activity in the presence of a single nucleotide triphosphate (Id.). In order for this procedure to work, the tag sequence must lack at least one of the 4 natural nucleotides such that polymerase degradation renders the entire tag sequence single stranded (Id.). The resulting product is contacted with beads to which tag complement is attached under conditions effective to permit hybridization of the tagged oligonucleotide to the complement tag (Id.). Ligase or some other chemical means is used to cross-link permanently the polynucleotide fragment to the bead (Id.). The product is then passed over a steptavidin coated slide and captured (Id.). The captured polynucleotides can then be sequenced (Id.). Thus, Brenner has nothing to do with a solution phase, ligase detection reaction based procedure, which utilizes a reusable addressable array, for detecting single nucleotide base differences. Moreover, as demonstrated in paragraph 17 of the Barany Declaration, Brenner's tag complement subunits are also very different from those of the present invention.

The prior art teaches away from the present invention in a number of other respects. On one hand, the art suggests that single stranded nucleic acids hybridize more efficiently than a double stranded product (Barany Declaration ¶ 38). However, the present invention utilizes a PCR product directly in an LDR procedure without modification (Id.). Existing array technology also suggests that capture probes be designed so that the matched or mismatched base is at or near the center of that probe (Barany Declaration ¶ 30). By contrast, in the present invention, the matched or mismatched base is not in the addressable array specific portion of the oligonucleotide probes which hybridize to the array capture probe. Finally, the background noise from mis-hybridization in the prior art arrays is too high to detect mutations in primary tumors (Barany Declaration ¶ 40). Thus, one of ordinary

skill in the art would not have expected the present invention to detect mutant DNA present in small amounts, as demonstrated *supra*.

It is thus apparent that the present invention is a significant advance over prior art LDR procedures and DNA array hybridization procedures. Accordingly, the prior art rejections made in the December 16, 1997, office action should be withdrawn.

Lastly, applicants have submitted revised versions of claims 12, 16, 18-20, 22, 26, 30, 34, and 81 further to overcome the outstanding rejection under 35 U.S.C. § 112 (2nd para.). With respect to claims 12, 16, 18-20, 22, 26, 30, and 34, the term "nearby" has been replaced with the phrase "overlapping oligonucleotide probe sets". Support for this limitation is found, for example, in Figure 5. Claim 81 now refers to oligonucleotide differences numerically instead of in terms of a percentage. See the comparison between zip11 and zip12 in the paragraph bridging pages 5-6 of the June 15, 1998, amendment for a discussion of the support for this in the present application.

For all these reasons, as well as those set forth in the June 26, 1998, amendment, applicants submit this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

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